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(54) DETECTION OF ENTEROVIRUS AND DISCRIMINATION OF THE SAME

(57)Abstract:

PURPOSE: To detect Picornaviridae such as Enterovirus, etc., by amplifying a Specific region of Enterovirus and detecting amplified gene DNA.

CONSTITUTION: An oligonucleotide (e.g. CTACTTTGGGTGTCCGTGTT) having complementarity to a common type part in the upstream of a gene region coding a part of 5'-non-translated region of Enterovirus, a part of VP4 and VP2 proteins, and an oligonucleotide (e.g. TGGTGGTGGGAAGTTGCCTGA) having complementarity to a common type part in the downstream are subjected to be primers of the PCR method. The amplified gene DNA is detected by polyacrylamide gel electrophoresis, etc.

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CLAIMS

[Claim(s)]

[Claim 1] (i) The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus, and some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The method of detecting the enterovirus characterized by amplifying the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus, and the human serum protein type of enterovirus, and detecting the (ii) this magnification gene DNA.

[Claim 2] (i) A part of 5'-untranslation region of an enterovirus separation stock with a strange human serum protein type The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus is amplified. This magnification gene DNA is solid-phase-ized to a microplate. A part of 5'-untranslation region of the epidemic enterovirus separation stock of known [human serum protein type / (ii)] The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. And carry out an indicator and it considers as the DNA probe for human serum protein type discernment. the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemic enterovirus separation stock, and the human serum protein type of enterovirus -- magnification -- (iii) this DNA probe -- the DNA solid phase-ized microplate of the above (i) -- in addition, the human serum protein type discernment approach of the enterovirus which is made to carry out hybridization under **** conditions, and is characterized by analyzing the class of joint probe.

[Claim 3] (i) The oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus and some ****4 and ****2 proteins is the following array (1).

CTACTTTGGGTGTCCGTGTT (1)

The oligonucleotide which comes out, has the base sequence shown and has a complementarity in a down-stream mold intersection is the following array (2).

TGGTGGTGGGAAGTTGCCTGA (2)

Detection or the discernment approach of claim 1 characterized by being the oligonucleotide which comes out and has the array shown, or the enterovirus of 2.

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]**[0001]**

[Industrial Application] This invention detects enterovirus to high sensitivity, and relates to the approach of identifying a human serum protein type.

[0002]

[Description of the Prior Art] It is difficult to presume the virus which the enterovirus (Enterovirus) belonging to the Picornaviridae (Picornaviridae) is classified into about 70 kinds of human serum protein types, and the rhinovirus (Rhinovirus) which similarly belongs to the Picornaviridae is classified into about 100 kinds of human serum protein types, shows a variegated infectious disease, and becomes a cause from a clinical manifestation. Therefore, separation identification of a virus is needed for deciding a pathogen. However, a current enterovirus separation method of identification separates a virus using cultivation, and the protection test is further needed for identification. And two - four weeks is required for the isolation culture of these viruses. The separation stock which the protection test which furthermore used the neutralization antiserum of a standard stock cannot human serum protein type judge appears frequently. This is considered for the gene of enterovirus to vary extremely in a nature at high speed, and production of the antiserum which always neutralizes a fresh separation stock is needed for these solutions. In chlamydia (Chlamydia), the approach of detecting for a short time, using a DNA probe as a direct detection method of the pathogen of an infectious disease is established. However, the detection sensitivity is low, and in enterovirus, in order that the amount of viruses required for the probe method may not be obtained from a patient specimen but the gene of enterovirus may vary to a high speed extremely still like previous statement, the difficulty of identification is expected with the oligo probe of a standard stock. High sensitivity and polymerase chain reaction method [Polymerase Chain Reaction which amplifies DNA specifically Law, ; which writes this as the "PCR method" below, after Saiki et al., Science, 230 volumes, p1350-1354, and 1985 reference] are developed the PCR method using a primer complementary to the base sequence of a 5'-untranslation region, and 5' -- by the PCR method using the primer which has a complementarity in the base sequence of the gene field which carries out the code of the ****4 and ****2 protein in - untranslation region [Rotbart. by which enterovirus is detected H. and 5.J. -- Clinical microbiology. and 28 438-442 (1990); Olive.D., M., 5 J. general Virology., 71, and 2141-2147 (1990) --]. However, these approaches cannot identify the human serum protein type of enterovirus, therefore enterovirus is detected in a higher precision and the approach of judging a human serum protein type is searched for.

[0003]

[Problem(s) to be Solved by the Invention] This invention aims at offer of the approach of judging the human serum protein type of enterovirus in a high precision while it can detect picornaviruses, such as enterovirus and rhinovirus, in a high precision.

[0004]

[Means for Solving the Problem] this invention persons have a specific base sequence in a part of 5'-untranslation region of enterovirus, and the human serum protein type of enterovirus. Detection [high

sensitivity picornaviruses /, such as enterovirus,] is possible by amplifying a field including the gene field which carries out the code of some ****4 and ****2 proteins, and detecting this magnification gene DNA, Furthermore, a human serum protein type uses a known epidemia enterovirus separation stock for this magnification gene DNA, and the same field as the above is combined under the produced DNA probe which amplified and carried out the indicator, and **** conditions. By detecting the joint indicator DNA and analyzing the class of united probe, it finds out that discernment of the highly precise human serum protein type of enterovirus is possible, and came to complete this invention.

[0005] According to this invention, in this way A part of 5'-untranslation region of 1. (i) enterovirus The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus is amplified. (ii) The method of detecting the enterovirus characterized by detecting this magnification gene DNA, 2. A part of 5'-untranslation region of an enterovirus separation stock with the strange (i) human serum protein type The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus is amplified. This magnification gene DNA is solid-phase-ized to a microplate. A part of 5'-untranslation region of the separation stock of the epidemia enterovirus of known [human serum protein type / (ii)] The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. And carry out an indicator and it considers as the DNA probe for human serum protein type discernment. the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus -- magnification -- A DNA probe is added to the DNA solid phase-ized microplate of the above (i). (iii) The human serum protein type discernment approach of the enterovirus which is made to carry out hybridization under **** conditions, and is characterized by analyzing the class of joint probe, 3. The oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of (i) enterovirus and some ****4 and ****2 proteins is the following array (1).

CTACTTTGGGTGTCCGTGTT (1)

The oligonucleotide which comes out, has the base sequence shown and has a complementarity in a down-stream mold intersection is the following array. TGGTGGTGAAGTTGCCTGA (2)

Detection or the discernment approach of of the above 1 or the enterovirus of 2 characterized by being the oligonucleotide which comes out and has the base sequence shown is offered.

[0006] Detection and the discernment approach of the enterovirus of this invention are further explained to a detail below. a particle symmetrical with the regular icosahedron of the ether resistance in which "picornavirus" does not have an envelope in this specification -- it is -- the diameter of 20-30nm -- it is -- a core -- single stranded RNA -- having -- the molecular weight of this RNA -- about 2.5×10^6 it is -- the virion which has infectivity and has the function of mRNA is meant. moreover, "enterovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- stable -- the buoyant density in the inside of CsCl -- 1.32-1.35g/cm³ it is -- virion is meant and the Coxsackie A group virus, the Coxsackie B group virus, echovirus, enterovirus, a poliovirus, etc. are included by this enterovirus. further -- "rhinovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- unstable -- the buoyant density in the inside of CsCl -- 1.38 - 1.40 g/cm² it is -- virion is meant. One description of this invention is to identify the human serum protein type of this enterovirus while detecting enterovirus by analyzing the class of probe which the human serum protein type increased a part of gene of the strange

enterovirus separation stock origin, made combine by the hybridization under the DNA probe and the **** conditions that magnification and the human serum protein type which carried out the indicator produced the same field from the gene of a known epidemia enterovirus separation stock, and combined. Such an approach enables it to identify the human serum protein type of enterovirus, while enterovirus is detectable in a high precision.

[0007] Since a close relationship [a human serum protein type / between those with about 70 sorts, and each human serum protein type], as for enterovirus, it is desirable to use the hybridization under the **** conditions which discernment of a human serum protein type is difficult, and are used by this invention on the occasion of discernment of a human serum protein type on the usual hybridization conditions. Here, the hybridization under **** conditions means the hybridization under existence of a formamide. Especially the abundance of the formamide in this hybridization condition usually has 40 - 60% of desirable within the limits 20 to 70%, and especially reaction temperature has desirable within the limits of 40-60 degrees C 40-70 degrees C. Although there is especially no limit in reaction time, within the limits of 1 - 24 hours is usually suitable. Although a standard stock and a separation stock (they are a vaccine stock and a decomposition stock in the case of a poliovirus) will be distinguished in the same human serum protein type and discernment of the human serum protein type of a separation stock is impossible in the hybridization under the above-mentioned **** conditions As a source of enterovirus gene DNA for the DNA probe creation for human serum protein type discernment The DNA probe for human serum protein type discernment by which the human serum protein type was created using the known epidemia enterovirus separation stock (namely, enterovirus stock which was in fashion and was separated within the past ten years) is used. It becomes discriminable [detection of each enterovirus, and a human serum protein type] by performing hybridization under the above-mentioned **** conditions, and analyzing a joint pattern.

[0008] Magnification of a gene field including the human serum protein type specific base sequence of enterovirus, i.e., "the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus", can be performed as follows. First, the isolation culture stock from clinical specimens, such as cerebrospinal fluid extracted at the time of a medical examination, and a clinical specimen and the human serum protein type by which subculture is carried out extract RNA from a known enterovirus standard stock etc. with a conventional method, and produces cDNA for this extract RNA using reverse transcriptase. The die length which includes the gene field which carries out the code of the 5'-untranslation region of enterovirus, and ****4 and ****2 for the oligonucleotide which has a human serum protein type specific base sequence, i.e., "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of an enterovirus separation stock and some ****4 and ****2 proteins, and a down-stream mold intersection", as a primer in this cDNA amplifies the gene DNA field of about 650 bases. the PCR method for which magnification of a gene is usually used -- [-- JP,61-274697,A, JP,62-281,A, 239 Sakai sScience(s), and p487-491 reference] can perform the detail of this PCR method easily.

[0009] On the occasion of magnification of a gene field including the human serum protein type specific base sequence of enterovirus, as an oligonucleotide which can be used as a primer The oligonucleotide which has a complementarity in the mold intersection of the upstream of a gene field including a human serum protein type specific base sequence, and a down-stream mold intersection, Namely, if "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus and some ****4 and ****2 proteins, and a down-stream mold intersection" is used for coincidence You may be what kind of oligonucleotide. It is appropriate to use as a primer the oligonucleotide which was specific to enterovirus, and set the high base sequence of similarity as the 5'-untranslation region (upstream mold intersection) and ****2 field (down-stream mold intersection) between seeds, and carried out chemosynthesis in them based on the base sequence based on desirable known human serum protein type specific base sequence data.

[0010] As the primer which carried out chemosynthesis, i.e., an oligonucleotide which has a complementarity in the mold intersection of the upstream of an enterovirus specific gene field, it is the following array (1).

CTACTTTGGGTGTCCGTGTT (1)

The oligonucleotide which has a complementarity in a down-stream mold intersection is the following array (2).

TGGTGGTGGGAAGTTGCCTGA (2)

It is more desirable to use the primer which comes out and has the base sequence shown. The chemosynthesis of the primer mentioned above is model 381-A, the known the nucleic-acid-biosynthesis machine usually used, for example, Applied Biosystem make, in itself. It can carry out easily with the solid phase synthesis method using a DNA synthesis machine etc. Like the above, polyacrylamide gel electrophoresis, agarose gel electrophoresis, etc. which are usually used can separate, and the gene field DNA including the human serum protein type specific base sequence of the enterovirus which was carried out and was amplified by the PCR method can be detected as a band, and, thereby, can check the gene DNA of the enterovirus origin. In addition, detection of the DNA band after electrophoresis can be dyed by the ethidium bromide, and UV irradiation can perform it easily.

[0011] DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus" obtained by the approach explained in full detail above is denatured with a conventional method, and it fixes on a microplate, and considers as Sample DNA (this may be called "solid phase-ized DNA" below). the approach same on the other hand as the above -- DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus" -- magnification -- and an indicator can be carried out and it can consider as the DNA probe for human serum protein type discernment. The indicator of this DNA probe for human serum protein type discernment changes and uses for Biotin dUTP a part of dTTP used for example, for a DNA magnification reaction, and can carry it out easily by performing DNA magnification.

[0012] the solid phase-ized DNA (sample DNA) above-mentioned after denaturing various kinds of DNA probes for human serum protein type discernment obtained in this way -- in addition, the human serum protein type of enterovirus used for preparation of solid phase-ized DNA (sample DNA) is discriminable by carrying out hybridization under said **** conditions, and detecting the class and amount of the DNA probe for human serum protein type discernment which were combined to solid phase-ized DNA using enzyme-labeling avidin etc.

[0013]

[Example] Hereafter, an example is given and this invention is further explained to a detail.

Example 1 It experimented using the 31 following kinds of human serum protein type picornavirus standard stocks by which subculture is carried out in detection of a picornavirus standard stock, and (Discernment A) use microorganism National Institute of Health of a human serum protein type. Each of such picornaviruses is standard stocks with which the human serum protein type is identified by the protection test which used the specific antiserum.

[0014]

[Table 1]

株名 (血清型)	略号	
コクサッキー A 群ウイルス	2 型	A 2
〃	3 〃	A 3
〃	4 〃	A 4
〃	8 〃	A 8
〃	9 〃	A 9
コクサッキー B 群ウイルス	1 型	B 1
〃	2 〃	B 2
〃	3 〃	B 3
〃	4 〃	B 4
〃	5 〃	B 5
〃	6 〃	B 6
エコーウイルス	3 型	E 3
〃	4 〃	E 4
〃	5 〃	E 5
〃	6 〃	E 6
〃	9 〃	E 9
〃	1 1 〃	E 1 1
〃	1 4 〃	E 1 4
〃	1 6 〃	E 1 6
〃	1 8 〃	E 1 8
〃	1 9 〃	E 1 9
〃	2 4 〃	E 2 4
〃	2 5 〃	E 2 5
〃	2 7 〃	E 2 7
〃	3 0 〃	E 3 0
エンテロウイルス	7 1 型	E 7 1
ポリオウイルス	1 型	P V 1
〃	2 〃	P V 2
〃	3 〃	P V 3
ライノウイルス	3 型	R H 3
〃	7 〃	R H 7

[0015] (B) The precipitate after settling extract above-mentioned each virus liquid of RNA by ultracentrifuge actuation by shoe cloth 15% It collected in Tris-EDTA, the phenol/chloroform extraction was performed, and ethanol precipitate was performed.

(C) cDNA which originates in each virus using a reverse transcriptase (Bthesda Research Laboratories) by using as mold each RNA obtained by the synthetic aforementioned (B) term of cDNA was compounded.

[0016] (D) the primer pair which can amplify the gene of the picornavirus of the synthetic aforementioned (A) term of the primer for PCR in common -- a human serum protein type -- the following array (1) which has a complementarity to each of a 5'-untranslation region and ****2 field based on the base sequence of the gene field which carries out the code of the ****4 and ****2 protein with a specific base sequence, and array (2) CTACTTTGGGTGTCCGTGTT (1)

TGGTGGTGGGAAGTTGCCTGA (2)

the primer of 20 bases shown by ***** -- phospho friend DAITO (Phosphoramidite) -- law -- Applied Biosystem make and model 381-A It compounded using the DNA synthesis machine, refined using the OPCTM cartridge, and was used as a primer of PCR.

[0017] (E) Magnification of the gene for solid phase-ized DNA preparation (sample DNA) (PCR) As reaction mixture, it is 10X. Buffer-solution (Reaction Buffer) 10microl for a reaction, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, dGTP, and dTTP; 1.25 mM each content) 16microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA compounded by the aforementioned (C) term Distilled water is added to 100ng-1microg and Taq polymerase (TAKARA SHUZO make) 1microl

(5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base acid was set up for 95-degree-C 30 seconds, it set up 1 minute and 45-degree-C base chain expanding process for the annealing process in 72-degree-C 2 minutes, and 1 cycle amplified 35 cycles of targets DNA using the amplification system (amplification system; SHITASU). This magnification gene was used as a sample DNA for solid-phase-izing.

[0018] (F) Magnification of the gene for DNA probe preparation for human serum protein type discernment (PCR)

It is 10X as reaction mixture. Buffer-solution (Reaction Buffer) 10microfor reaction 1, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, and dGTP; 1.25 mM(s)each dTTP; 0.94 mM) 16microl, Biotin-11-dUTP(Enzo Diagnostics)16.7microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA100ng-1microg compounded by the aforementioned (C) term It reaches, distilled water is added to Taq polymerase (TAKARA SHUZO) 1microl (5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base chain is set up for 95-degree-C 30 seconds, it sets up 1 minute and 45-degree-C base chain expanding process for an annealing process in 72-degree-C 2 minutes, and 1 cycle is amplification. 35 cycles of targets DNA were amplified using the system (SHITASU). The gene DNA by which the indicator was carried out by this biotin was used as a DNA probe for human serum protein type discernment.

[0019] (G) The ethidium bromide was added to agarose gel of 3.0% of checks of the magnification gene DNA by gel electrophoresis ml 0.5microg /, and electrophoresis of DNA amplified by the above (E) and the (F) term was performed. 254nm ultraviolet rays were irradiated after migration, the coloring reaction of the ethidium bromide detected the DNA band, and the target DNA band of about 650 bases originating in the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in the part and human serum protein type of a 5'-untranslation region of enterovirus was checked.

(H) The gene DNA amplified by purification of Magnification DNA, the density measurement above (E), and the (F) term was settled after an extract and using ethanol under a phenol/chloroform, it collected, and concentration was computed with the absorbance of 260nm.

[0020] (I) Plate hybridization microplate solid phase technique (Inouye Hondo.J.Cli.Microbiol.28:1469.1990) It carried out by the strange method. They are 1.5M NaCl, 10mM sodium phosphate, and 10mM after thermal denaturation and about 50ng / 100microl/well in the sample DNA refined by the above-mentioned (H) term. It is a microplate (NUNC-IMMUNO PLATE MAXISORP F96) under EDTA existence. It solid-phase-ized in 37-degree-C 2 hours. This was washed 3 times by PBS-Tween 20, and the unreacted sample DNA was removed. Hybridization performed 1.25ng / 50 degree C of 100microl/well for the DNA probe for human serum protein type discernment refined by the aforementioned (H) term to said microplate after thermal denaturation for 8 hours under 50% formamide, 0.75MNaCl, 0.1%Tween 20, and Salmon sperm 50microg/ml existence. The microplate was washed 3 times by PBS-Tween 20 after hybridization, and the DNA probe for unreacted human serum protein type discernment was removed. next, 1:1,000 diluent (1%BSA, 0.1% Triton X-100, and PBS-Tween 20) of peroxidase-labeling streptoavidin -- dropping -- it was made to react for room temperature 2 hours It is after 3 times washing, 0.012%H₂ O₂ and 0.04% alt.phenylenediamine, and 0.05/0.024M at PBS-Tween 20 about a microplate again. An sodium phosphate-citric acid (pH5.0) is made to react in the state of protection from light at a room temperature in addition for 30 minutes so that it may become 100microl/well, and it is 4 Ns. 50micro l/well of sulfuric acids was added, and the reaction was stopped. The absorbance (OD) was measured for the amount of coloring of the microplate produced by the reaction on the wavelength of 492nm using the microplate reader (Biorad make). It asked for the binding fraction (%) of the DNA probe for human serum protein type discernment from the absorbance of each microplate as follows.

Binding fraction (%) =(OD value of hybridization of solid phase-ized DNA [of the OD value / same human serum protein type virus origin of the hybridization of a solid phase-ized DNA of the human serum protein type virus origin and the DNA probe for discernment which are different in **], and

DNA probe for discernment) x100.

The result is shown in the 1st table. In addition, each null column in the 1st table is the value of less than 10% of association.

[0021]

[Table 2]

標準株の型鑑別（株）

[illegible]

[0022] (J) The amplified target DNA band was detected by the gel electrophoresis after PCR about results and all the **** picornavirus standard stocks for consideration (31 shares). Moreover, as a result of performing plate hybridization, the cross reaction was not accepted between the magnification DNA of each human serum protein type origin as the joint pattern shown in the 1st table. It became clear from this joint pattern for detection of enterovirus and discernment of each human serum protein type to be possible.

[0023] Example 2 It experimented using the enterovirus separation stock with which it dissociated from the patient of (Discernment A) use microorganism following of detection of an enterovirus separation stock, and a human serum protein type, and the human serum protein type was identified by the protection test using the specific antiserum, and the standard stock of an example 1.

(1) Enterovirus separation stock [Table 3]

株名 (血清型)	分離時期
コクサッキー A 群ウイルス 4 型 (A 4)	
1 1 5 5 / 7 2	1 9 7 2 年
1 3 6 1 / 8 2	1 9 8 2 年
0 2 6 9 / 8 4	1 9 8 4 年
0 0 2 5 / 8 6	1 9 8 6 年
0 0 2 3 / 8 7	1 9 8 7 年
0 4 0 6 / 8 9	1 9 8 9 年
0 3 1 3 / 9 1	1 9 9 1 年
エコーウイルス 1 1 型 (E 1 1)	
1 0 3 6 / 7 1	1 9 7 1 年
1 1 8 3 / 7 7	1 9 7 7 年
1 1 4 9 / 8 7	1 9 8 7 年
3 1 3 7 / 8 1	1 9 8 1 年
1 3 0 3 / 8 3	1 9 8 3 年
0 7 9 8 / 8 4	1 9 8 4 年
0 4 0 0 / 8 5	1 9 8 5 年
0 1 0 7 / 9 0	1 9 9 0 年
エンテロウイルス 7 1 型 (E 7 1)	
ナゴヤ / 7 0	1 9 7 0 年
3 0 5 9 / 7 8	1 9 7 8 年
3 3 5 9 / 8 3	1 9 8 3 年
4 1 3 2 / 8 5	1 9 8 5 年
2 3 6 a / 8 6	1 9 8 6 年
2 3 6 c / 8 6	1 9 8 6 年
0 2 5 3 / 8 6	1 9 8 6 年
2 5 8 7 / 8 9	1 9 8 9 年
4 0 9 4 / 9 0	1 9 9 0 年

[0024] (2) Standard stock [Table 4]

コクサッキー A 群ウイルス	4 型 (A 4)
コクサッキー B 群ウイルス	2 (B 2)
〃	3 (B 3)
〃	5 (B 5)
エコーウイルス	9 (E 9)
〃	1 1 (E 1 1)
〃	3 0 (E 3 0)
エンテロウイルス	7 1 (E 7 1)
ポリオウイルス	3 (P V 3)

[0025] (B) the experiment approach and the approach of each virus of the result above to the example 1

given in (B) term -- RNA -- extracting -- an approach given in (** C) term -- every -- cDNA was compounded. Furthermore, as a result of amplifying the gene for solid phase-ized DNA preparation by the approach given in (** E) term, amplifying the gene for DNA probe preparation for human serum protein type discernment by the approach given in (** F) term and performing gel electrophoresis given in (** G) term about these magnification genes DNA, the magnification gene DNA band originating in all the used stocks has been checked. After refining these magnification gene DNA by the approach given in (** H) term and performing density measurement, plate hybridization was carried out like (** I) term publication, and the binding fraction (%) of each probe was computed. The result is shown in the 2nd table - the 4th table. In addition, the binding fraction of the null column of front Naka is 10% or less of value.

[0026]

[Table 5]

第 2 表

コクサッキーA群ウイルス4型（A4）分離株の型鑑別（結合率：％）

			血清型識別用DNAプローブ						
			1155/72	1361/82	0269/84	0025/86	0023/87	0406/89	0313/91
固 相 化 D N A	A 4 分 離 株	1155/72	100						
		1361/82		100					
		0269/84			100	63	50	50	58
		0025/86			81	100	60	43	50
		0023/87			50	44	100	36	33
		0406/89			56	44	36	100	100
		0313/91			56	44	29	79	100
		標準株							
		A4							100
		B2							
		B3							
		B5							
		E9							
		E11							
		E30							
		E71							
		PV3							

[0027]

[Table 6]

第3表
エコーウイルス11型(E11)分離株の型鑑別(結合率:%)

		血清型識別用DNAプロープ									
		1036/71	1183/77	1149/78	3137/81	1303/83	0798/84	0400/85	0107/90	標準株E11	
固相化DNA	E11分離株	100	100		37	37		33	23		
				100	20	22		20			
		43		23	100	111	103	117	92		
		33		20	73	100	76	108	81		
		20			93	100	100	104	81		
		33		20	80	93	76	100	77		
		23			67	78	62	79	100		
標準株		A4	B2	B3	B5	E9	E11	E30	E71	PV3	100

[0028]
[Table 7]

第 4 表

		血清型識別用 DNA プローブ									
		71ヤ / 70	3059/78	3359/83	4132/85	236a/86	236c/86	0253/86	2587/89	4094/90	標準株E71
固相化 DNA	71ヤ / 70	100	110	100	84	98	90	89			
	E 3059/78	75	100	64	105	75	62	63			
	71 3359/83	82	85	100	100	82	83	85	22		
	分離 4132/85	79	95	73	100	71	69	63	26	38	
	株 236a/86	82	90	91	84	100	97	93			
	236c/86	89	100	100	58	104	100	100			
A	0253/86	82	90	95	84	104	93	100			
	2587/89			32	37				100	119	
	4094/90				37				78	100	
											100
標準株		A4	B2	B3	B5	E9	E11	E30	E71	PV3	

[0029] The cross reaction was not accepted between the same human serum protein types between solid phase-ized DNA of the standard stock origin of all the DNA probes and each human serum protein types of the used enterovirus separation stock a passage clear from the joint pattern shown in the 2nd table - the 4th table. On the other hand, about the separation stock in each human serum protein type, the high cross reaction was accepted on the epidemic viral isolation stock (between the same human serum protein types) separated within about ten years. The gene field where a human serum protein type has a

specific base sequence in said human serum protein type of a known epidemic enterovirus separation stock (stock separated within about ten years) from the above result was amplified, and when performing hybridization using the DNA probe for human serum protein type discernment obtained, it became clear for detection of epidemic enterovirus and discernment of a human serum protein type to be easily possible.

[Translation done.]

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TECHNICAL FIELD

[Industrial Application] This invention detects enterovirus to high sensitivity, and relates to the approach of identifying a human serum protein type.

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PRIOR ART

[Description of the Prior Art] It is difficult to presume the virus which the enterovirus (Enterovirus) belonging to the Picornaviridae (Picornaviridae) is classified into about 70 kinds of human serum protein types, and the rhinovirus (Rhinovirus) which similarly belongs to the Picornaviridae is classified into about 100 kinds of human serum protein types, shows a variegated infectious disease, and becomes a cause from a clinical manifestation. Therefore, separation identification of a virus is needed for deciding a pathogen. However, a current enterovirus separation method of identification separates a virus using cultivation, and the protection test is further needed for identification. And two - four weeks is required for the isolation culture of these viruses. The separation stock which the protection test which furthermore used the neutralization antiserum of a standard stock cannot human serum protein type judge appears frequently. This is considered for the gene of enterovirus to vary extremely in a nature at high speed, and production of the antiserum which always neutralizes a fresh separation stock is needed for these solutions. In chlamydia (Chlamydia), the approach of detecting for a short time, using a DNA probe as a direct detection method of the pathogen of an infectious disease is established. However, the detection sensitivity is low, and in enterovirus, in order that the amount of viruses required for the probe method may not be obtained from a patient specimen but the gene of enterovirus may vary to a high speed extremely still like previous statement, the difficulty of identification is expected with the oligo probe of a standard stock. High sensitivity and polymerase chain reaction method [Polymerase Chain Reaction which amplifies DNA specifically Law, ; which writes this as the "PCR method" below, after Saiki et al., Science, 230 volumes, p1350-1354, and 1985 reference] are developed the PCR method using a primer complementary to the base sequence of a 5'-untranslation region, and 5' -- by the PCR method using the primer which has a complementarity in the base sequence of the gene field which carries out the code of the ****4 and ****2 protein in - untranslation region [Rotbart. by which enterovirus is detected H. and 5.J. -- Clinical microbiology. and 28 438-442 (1990); Olive.D., M., 5 J.general Virology., 71, and 2141-2147 (1990) --]. However, these approaches cannot identify the human serum protein type of enterovirus, therefore enterovirus is detected in a higher precision and the approach of judging a human serum protein type is searched for.

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MEANS

[Means for Solving the Problem] this invention persons have a specific base sequence in a part of 5'-untranslation region of enterovirus, and the human serum protein type of enterovirus. Detection [high sensitivity picornaviruses /, such as enterovirus,] is possible by amplifying a field including the gene field which carries out the code of some ****4 and ****2 proteins, and detecting this magnification gene DNA, Furthermore, a human serum protein type uses a known epidemia enterovirus separation stock for this magnification gene DNA, and the same field as the above is combined under the produced DNA probe which amplified and carried out the indicator, and **** conditions. By detecting the joint indicator DNA and analyzing the class of united probe, it finds out that discernment of the highly precise human serum protein type of enterovirus is possible, and came to complete this invention. [0005] According to this invention, in this way A part of 5'-untranslation region of 1. (i) enterovirus The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus is amplified. (ii) The method of detecting the enterovirus characterized by detecting this magnification gene DNA, 2. A part of 5'-untranslation region of an enterovirus separation stock with the strange (i) human serum protein type The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. The gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus is amplified. This magnification gene DNA is solid-phase-ized to a microplate. A part of 5'-untranslation region of the separation stock of the epidemia enterovirus of known [human serum protein type / (ii)] The oligonucleotide which has a complementarity is used for the mold intersection of the upstream of the gene field which carries out the code of some ****4 and ****2 proteins, and a down-stream mold intersection as a primer. And carry out an indicator and it considers as the DNA probe for human serum protein type discernment. the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus -- magnification -- A DNA probe is added to the DNA solid phase-ized microplate of the above (i). (iii) The human serum protein type discernment approach of the enterovirus which is made to carry out hybridization under **** conditions, and is characterized by analyzing the class of joint probe, 3. The oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of (i) enterovirus and some ****4 and ****2 proteins is the following array (1).

CTACTTTGGGTGTCCGTGTT (1)

The oligonucleotide which comes out, has the base sequence shown and has a complementarity in a

down-stream mold intersection is the following array. TGGTGGTGGGAAGTTGCCTGA (2)

Detection or the discernment approach of the above 1 or the enterovirus of 2 characterized by being the oligonucleotide which comes out and has the base sequence shown is offered.

[0006] Detection and the discernment approach of the enterovirus of this invention are further explained to a detail below. a particle symmetrical with the regular icosahedron of the ether resistance in which "picornavirus" does not have an envelope in this specification -- it is -- the diameter of 20-30nm -- it is -- a core -- single stranded RNA -- having -- the molecular weight of this RNA -- about 2.5×10^6 it is -- the virion which has infectivity and has the function of mRNA is meant. moreover, "enterovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- stable -- the buoyant density in the inside of CsCl -- 1.32-1.35g/cm³ it is -- virion is meant and the Cocksackie A group virus, the Cocksackie B group virus, echovirus, enterovirus, a poliovirus, etc. are included by this enterovirus. further -- "rhinovirus" -- the above-mentioned Picornaviridae -- belonging -- and pH3.0 -- unstable -- the buoyant density in the inside of CsCl -- 1.38 - 1.40 g/cm² it is -- virion is meant. One description of this invention is to identify the human serum protein type of this enterovirus while detecting enterovirus by analyzing the class of probe which the human serum protein type increased a part of gene of the strange enterovirus separation stock origin, made combine by the hybridization under the DNA probe and the **** conditions that magnification and the human serum protein type which carried out the indicator produced the same field from the gene of a known epidemia enterovirus separation stock, and combined. Such an approach enables it to identify the human serum protein type of enterovirus, while enterovirus is detectable in a high precision.

[0007] Since a close relationship [a human serum protein type / between those with about 70 sorts, and each human serum protein type], as for enterovirus, it is desirable to use the hybridization under the **** conditions which discernment of a human serum protein type is difficult, and are used by this invention on the occasion of discernment of a human serum protein type on the usual hybridization conditions. Here, the hybridization under **** conditions means the hybridization under existence of a formamide. Especially the abundance of the formamide in this hybridization condition usually has 40 - 60% of desirable within the limits 20 to 70%, and especially reaction temperature has desirable within the limits of 40-60 degrees C 40-70 degrees C. Although there is especially no limit in reaction time, within the limits of 1 - 24 hours is usually suitable. Although a standard stock and a separation stock (they are a vaccine stock and a decomposition stock in the case of a poliovirus) will be distinguished in the same human serum protein type and discernment of the human serum protein type of a separation stock is impossible in the hybridization under the above-mentioned **** conditions As a source of enterovirus gene DNA for the DNA probe creation for human serum protein type discernment The DNA probe for human serum protein type discernment by which the human serum protein type was created using the known epidemia enterovirus separation stock (namely, enterovirus stock which was in fashion and was separated within the past ten years) is used. It becomes discriminable [detection of each enterovirus, and a human serum protein type] by performing hybridization under the above-mentioned **** conditions, and analyzing a joint pattern.

[0008] Magnification of a gene field including the human serum protein type specific base sequence of enterovirus, i.e., "the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in a part of 5'-untranslation region of enterovirus and the human serum protein type of enterovirus", can be performed as follows. First, the isolation culture stock from clinical specimens, such as cerebrospinal fluid extracted at the time of a medical examination, and a clinical specimen and the human serum protein type by which subculture is carried out extract RNA from a known enterovirus standard stock etc. with a conventional method, and produces cDNA for this extract RNA using reverse transcriptase. The die length which includes the gene field which carries out the code of the 5'-untranslation region of enterovirus, and ****4 and ****2 for the oligonucleotide which has a human serum protein type specific base sequence, i.e., "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of an enterovirus separation stock and some ****4 and ****2 proteins, and a down-stream mold intersection", as a primer in this cDNA amplifies the gene DNA field of about

650 bases. the PCR method for which magnification of a gene is usually used -- [-- JP,61-274697,A, JP,62-281,A, 239 Sakai sScience(s), and p487-491 reference] can perform the detail of this PCR method easily.

[0009] On the occasion of magnification of a gene field including the human serum protein type specific base sequence of enterovirus, as an oligonucleotide which can be used as a primer The oligonucleotide which has a complementarity in the mold intersection of the upstream of a gene field including a human serum protein type specific base sequence, and a down-stream mold intersection, Namely, if "the oligonucleotide which has a complementarity in the mold intersection of the upstream of the gene field which carries out the code of a part of 5'-untranslation region of enterovirus and some ****4 and ****2 proteins, and a down-stream mold intersection" is used for coincidence You may be what kind of oligonucleotide. It is appropriate to use as a primer the oligonucleotide which was specific to enterovirus, and set the high base sequence of similarity as the 5'-untranslation region (upstream mold intersection) and ****2 field (down-stream mold intersection) between seeds, and carried out chemosynthesis in them based on the base sequence based on desirable known human serum protein type specific base sequence data.

[0010] As the primer which carried out chemosynthesis, i.e., an oligonucleotide which has a complementarity in the mold intersection of the upstream of an enterovirus specific gene field, it is the following array (1).

CTACTTTGGGTGTCCGTGTT (1)

The oligonucleotide which has a complementarity in a down-stream mold intersection is the following array (2).

TGGTGGTGGGAAGTTGCCTGA (2)

It is more desirable to use the primer which comes out and has the base sequence shown. The chemosynthesis of the primer mentioned above is model 381-A, the known the nucleic-acid-biosynthesis machine usually used, for example, Applied Biosystem make, in itself. It can carry out easily with the solid phase synthesis method using a DNA synthesis machine etc. Like the above, polyacrylamide gel electrophoresis, agarose gel electrophoresis, etc. which are usually used can separate, and the gene field DNA including the human serum protein type specific base sequence of the enterovirus which was carried out and was amplified by the PCR method can be detected as a band, and, thereby, can check the gene DNA of the enterovirus origin. In addition, detection of the DNA band after electrophoresis can be dyed by the ethidium bromide, and UV irradiation can perform it easily.

[0011] DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a strange enterovirus separation stock and the human serum protein type of enterovirus" obtained by the approach explained in full detail above is denatured with a conventional method, and it fixes on a microplate, and considers as Sample DNA (this may be called "solid phase-ized DNA" below). the approach same on the other hand as the above -- DNA of "the gene field which carries out the code of some ****4 and ****2 proteins with which a human serum protein type has a specific base sequence in a part of 5'-untranslation region of a known epidemia enterovirus separation stock, and the human serum protein type of enterovirus" -- magnification -- and an indicator can be carried out and it can consider as the DNA probe for human serum protein type discernment. The indicator of this DNA probe for human serum protein type discernment changes and uses for Biotin dUTP a part of dTTP used for example, for a DNA magnification reaction, and can carry it out easily by performing DNA magnification.

[0012] the solid phase-ized DNA (sample DNA) above-mentioned after denaturing various kinds of DNA probes for human serum protein type discernment obtained in this way -- in addition, the human serum protein type of enterovirus used for preparation of solid phase-ized DNA (sample DNA) is discriminable by carrying out hybridization under said **** conditions, and detecting the class and amount of the DNA probe for human serum protein type discernment which were combined to solid phase-ized DNA using enzyme-labeling avidin etc.

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EXAMPLE

[Example] Hereafter, an example is given and this invention is further explained to a detail.

Example 1 It experimented using the 31 following kinds of human serum protein type picornavirus standard stocks by which subculture is carried out in detection of a picornavirus standard stock, and (Discernment A) use microorganism National Institute of Health of a human serum protein type. Each of such picornaviruses is standard stocks with which the human serum protein type is identified by the protection test which used the specific antiserum.

[0014]

[Table 1]

株名 (血清型)		略号
コクサッキー A 群ウイルス	2 型	A 2
〃	3 〃	A 3
〃	4 〃	A 4
〃	8 〃	A 8
〃	9 〃	A 9
コクサッキー B 群ウイルス	1 型	B 1
〃	2 〃	B 2
〃	3 〃	B 3
〃	4 〃	B 4
〃	5 〃	B 5
〃	6 〃	B 6
エコーウイルス	3 型	E 3
〃	4 〃	E 4
〃	5 〃	E 5
〃	6 〃	E 6
〃	9 〃	E 9
〃	1 1 〃	E 1 1
〃	1 4 〃	E 1 4
〃	1 6 〃	E 1 6
〃	1 8 〃	E 1 8
〃	1 9 〃	E 1 9
〃	2 4 〃	E 2 4
〃	2 5 〃	E 2 5
〃	2 7 〃	E 2 7
〃	3 0 〃	E 3 0
エンテロウイルス	7 1 型	E 7 1
ポリオウイルス	1 型	P V 1
〃	2 〃	P V 2
〃	3 〃	P V 3
ライノウイルス	3 型	R H 3
〃	7 〃	R H 7

[0015] (B) The precipitate after settling extract above-mentioned each virus liquid of RNA by ultracentrifuge actuation by shoe cloth 15% It collected in Tris-EDTA, the phenol/chloroform extraction was performed, and ethanol precipitate was performed.

(C) cDNA which originates in each virus using a reverse transcriptase (Bthesda Research Laboratories) by using as mold each RNA obtained by the synthetic aforementioned (B) term of cDNA was compounded.

[0016] (D) the primer pair which can amplify the gene of the picornavirus of the synthetic aforementioned (A) term of the primer for PCR in common -- a human serum protein type -- the following array (1) which has a complementarity to each of a 5'-untranslation region and ****2 field based on the base sequence of the gene field which carries out the code of the ****4 and ****2 protein with a specific base sequence, and array (2) CTACTTTGGGTGTCCGTGTT (1) TGGTGGTGGAAAGTTGCCTGA (2)

the primer of 20 bases shown by ***** -- phospho friend DAITO (Phosphoramidite) -- law -- Applied Biosystem make and model 381-A It compounded using the DNA synthesis machine, refined using the OPCTM cartridge, and was used as a primer of PCR.

[0017] (E) Magnification of the gene for solid phase-ized DNA preparation (sample DNA) (PCR) As reaction mixture, it is 10X. Buffer-solution (Reaction Buffer) 10microl for a reaction, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, dGTP, and dTTP; 1.25 mM each content) 16microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA compounded by the aforementioned (C) term Distilled water is added to 100ng-1microg and Taq polymerase (TAKARA SHUZO make) 1microl (5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base acid was set up for 95-degree-C 30 seconds, it set up 1 minute and 45-degree-C base chain expanding process for the annealing process in 72-degree-C 2 minutes, and 1 cycle amplified 35 cycles of targets DNA using the amplification system (amplification system; SHITASU). This magnification gene was used as a sample DNA for solid-phase-izing.

[0018] (F) Magnification of the gene for DNA probe preparation for human serum protein type discernment (PCR)

It is 10X as reaction mixture. Buffer-solution (Reaction Buffer) 10microfor reaction 1, Deoxy nucleotide 3-phosphoric-acid mixed liquor (dATP, dCTP, and dGTP; 1.25 mM(s)each dTTP; 0.94 mM) 16microl, Biotin-11-dUTP(Enzo Diagnostics)16.7microl, The above-mentioned synthetic primer (1) (50microM) 2.0microl and the above-mentioned synthetic primer (2) (50microM) 2.0microl, Picornavirus cDNA100ng-1microg compounded by the aforementioned (C) term It reaches, distilled water is added to Taq polymerase (TAKARA SHUZO) 1microl (5Unit), and it is total 100microl. What was carried out was prepared. The denaturation process of a base chain is set up for 95-degree-C 30 seconds, it sets up 1 minute and 45-degree-C base chain expanding process for an annealing process in 72-degree-C 2 minutes, and 1 cycle is amplification. 35 cycles of targets DNA were amplified using the system (SHITASU). The gene DNA by which the indicator was carried out by this biotin was used as a DNA probe for human serum protein type discernment.

[0019] (G) The ethidium bromide was added to agarose gel of 3.0% of checks of the magnification gene DNA by gel electrophoresis ml 0.5microg /, and electrophoresis of DNA amplified by the above (E) and the (F) term was performed. 254nm ultraviolet rays were irradiated after migration, the coloring reaction of the ethidium bromide detected the DNA band, and the target DNA band of about 650 bases originating in the gene field which carries out the code of some ****4 and ****2 proteins which have a specific base sequence in the part and human serum protein type of a 5'-untranslation region of enterovirus was checked.

(H) The gene DNA amplified by purification of Magnification DNA, the density measurement above (E), and the (F) term was settled after an extract and using ethanol under a phenol/chloroform, it collected, and concentration was computed with the absorbance of 260nm.

[0020] (I) Plate hybridization microplate solid phase technique (Inouye Hondo.J.Cli.Microbiol.28:1469.1990) It carried out by the strange method. They are 1.5M NaCl, 10mM

sodium phosphate, and 10mM after thermal denaturation and about 50ng / 100microl/well in the sample DNA refined by the above-mentioned (H) term. It is a microplate (NUNC-IMMUNO PLATE MAXISORP F96) under EDTA existence. It solid-phase-ized in 37-degree-C 2 hours. This was washed 3 times by PBS-Tween 20, and the unreacted sample DNA was removed. Hybridization performed 1.25ng / 50 degree C of 100microl/well for the DNA probe for human serum protein type discernment refined by the aforementioned (H) term to said microplate after thermal denaturation for 8 hours under 50% formamide, 0.75MNaCl, 0.1%Tween 20, and Salmon sperm 50microg/ml existence. The microplate was washed 3 times by PBS-Tween 20 after hybridization, and the DNA probe for unreacted human serum protein type discernment was removed. next, 1:1,000 diluent (1%BSA, 0.1% Triton X-100, and PBS-Tween 20) of peroxidase-labeling streptoavidin -- dropping -- it was made to react for room temperature 2 hours It is after 3 times washing, 0.012%H₂O₂ and 0.04% alt.phenylenediamine, and 0.05/0.024M at PBS-Tween 20 about a microplate again. An sodium phosphate-citric acid (pH5.0) is made to react in the state of protection from light at a room temperature in addition for 30 minutes so that it may become 100microl/well, and it is 4 Ns. 50micro l/well of sulfuric acids was added, and the reaction was stopped. The absorbance (OD) was measured for the amount of coloring of the microplate produced by the reaction on the wavelength of 492nm using the microplate reader (Biorad make). It asked for the binding fraction (%) of the DNA probe for human serum protein type discernment from the absorbance of each microplate as follows.

Binding fraction (%) =(OD value of hybridization of solid phase-ized DNA [of the OD value / same human serum protein type virus origin of the hybridization of a solid phase-ized DNA of the human serum protein type virus origin and the DNA probe for discernment which are different in **], and DNA probe for discernment) x100.

The result is shown in the 1st table. In addition, each null column in the 1st table is the value of less than 10% of association.

[0021]

[Table 2]

第 1 表

		電 機 測 定 用 器										電 機 測 定 用 器																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
		A2	A3	A4	A5	A6	A7	A8	A9	B1	B2	B3	B4	B5	B6	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	E23	E24	E25	E26	E27	E28	E29	E30	E31	E32	E33	E34	E35	E36	E37	E38	E39	E40	E41	E42	E43	E44	E45	E46	E47	E48	E49	E50	E51	E52	E53	E54	E55	E56	E57	E58	E59	E60	E61	E62	E63	E64	E65	E66	E67	E68	E69	E70	E71	E72	E73	E74	E75	E76	E77	E78	E79	E80	E81	E82	E83	E84	E85	E86	E87	E88	E89	E90	E91	E92	E93	E94	E95	E96	E97	E98	E99	E100	E101	E102	E103	E104	E105	E106	E107	E108	E109	E110	E111	E112	E113	E114	E115	E116	E117	E118	E119	E120	E121	E122	E123	E124	E125	E126	E127	E128	E129	E130	E131	E132	E133	E134	E135	E136	E137	E138	E139	E140	E141	E142	E143	E144	E145	E146	E147	E148	E149	E150	E151	E152	E153	E154	E155	E156	E157	E158	E159	E160	E161	E162	E163	E164	E165	E166	E167	E168	E169	E170	E171	E172	E173	E174	E175	E176	E177	E178	E179	E180	E181	E182	E183	E184	E185	E186	E187	E188	E189	E190	E191	E192	E193	E194	E195	E196	E197	E198	E199	E200	E201	E202	E203	E204	E205	E206	E207	E208	E209	E210	E211	E212	E213	E214	E215	E216	E217	E218	E219	E220	E221	E222	E223	E224	E225	E226	E227	E228	E229	E230	E231	E232	E233	E234	E235	E236	E237	E238	E239	E240	E241	E242	E243	E244	E245	E246	E247	E248	E249	E250	E251	E252	E253	E254	E255	E256	E257	E258	E259	E260	E261	E262	E263	E264	E265	E266	E267	E268	E269	E270	E271	E272	E273	E274	E275	E276	E277	E278	E279	E280	E281	E282	E283	E284	E285	E286	E287	E288	E289	E290	E291	E292	E293	E294	E295	E296	E297	E298	E299	E300	E301	E302	E303	E304	E305	E306	E307	E308	E309	E310	E311	E312	E313	E314	E315	E316	E317	E318	E319	E320	E321	E322	E323	E324	E325	E326	E327	E328	E329	E330	E331	E332	E333	E334	E335	E336	E337	E338	E339	E340	E341	E342	E343	E344	E345	E346	E347	E348	E349	E350	E351	E352	E353	E354	E355	E356	E357	E358	E359	E360	E361	E362	E363	E364	E365	E366	E367	E368	E369	E370	E371	E372	E373	E374	E375	E376	E377	E378	E379	E380	E381	E382	E383	E384	E385	E386	E387	E388	E389	E390	E391	E392	E393	E394	E395	E396	E397	E398	E399	E400	E401	E402	E403	E404	E405	E406	E407	E408	E409	E410	E411	E412	E413	E414	E415	E416	E417	E418	E419	E420	E421	E422	E423	E424	E425	E426	E427	E428	E429	E430	E431	E432	E433	E434	E435	E436	E437	E438	E439	E440	E441	E442	E443	E444	E445	E446	E447	E448	E449	E450	E451	E452	E453	E454	E455	E456	E457	E458	E459	E460	E461	E462	E463	E464	E465	E466	E467	E468	E469	E470	E471	E472	E473	E474	E475	E476	E477	E478	E479	E480	E481	E482	E483	E484	E485	E486	E487	E488	E489	E490	E491	E492	E493	E494	E495	E496	E497	E498	E499	E500	E501	E502	E503	E504	E505	E506	E507	E508	E509	E510	E511	E512	E513	E514	E515	E516	E517	E518	E519	E520	E521	E522	E523	E524	E525	E526	E527	E528	E529	E530	E531	E532	E533	E534	E535	E536	E537	E538	E539	E540	E541	E542	E543	E544	E545	E546	E547	E548	E549	E550	E551	E552	E553	E554	E555	E556	E557	E558	E559	E560	E561	E562	E563	E564	E565	E566	E567	E568	E569	E570	E571	E572	E573	E574	E575	E576	E577	E578	E579	E580	E581	E582	E583	E584	E585	E586	E587	E588	E589	E590	E591	E592	E593	E594	E595	E596	E597	E598	E599	E600	E601	E602	E603	E604	E605	E606	E607	E608	E609	E610	E611	E612	E613	E614	E615	E616	E617	E618	E619	E620	E621	E622	E623	E624	E625	E626	E627	E628	E629	E630	E631	E632	E633	E634	E635	E636	E637	E638	E639	E640	E641	E642	E643	E644	E645	E646	E647	E648	E649	E650	E651	E652	E653	E654	E655	E656	E657	E658	E659	E660	E661	E662	E663	E664	E665	E666	E667	E668	E669	E670	E671	E672	E673	E674	E675	E676	E677	E678	E679	E680	E681	E682	E683	E684	E685	E686	E687	E688	E689	E690	E691	E692	E693	E694	E695	E696	E697	E698	E699	E700	E701	E702	E703	E704	E705	E706	E707	E708	E709	E710	E711	E712	E713	E714	E715	E716	E717	E718	E719	E720	E721	E722	E723	E724	E725	E726	E727	E728	E729	E730	E731	E732	E733	E734	E735	E736	E737	E738	E739	E740	E741	E742	E743	E744	E745	E746	E747	E748	E749	E750	E751	E752	E753	E754	E755	E756	E757	E758	E759	E760	E761	E762	E763	E764	E765	E766	E767	E768	E769	E770	E771	E772	E773	E774	E775	E776	E777	E778	E779	E780	E781	E782	E783	E784	E785	E786	E787	E788	E789	E790	E791	E792	E793	E794	E795	E796	E797	E798	E799	E800	E801	E802	E803	E804	E805	E806	E807	E808	E809	E810	E811	E812	E813	E814	E815	E816	E817	E818	E819	E820	E821	E822	E823	E824	E825	E826	E827	E828	E829	E830	E831	E832	E833	E834	E835	E836	E837	E838	E839	E840	E841	E842	E843	E844	E845	E846	E847	E848	E849	E850	E851	E852	E853	E854	E855	E856	E857	E858	E859	E860	E861	E862	E863	E864	E865	E866	E867	E868	E869	E870	E871	E872	E873	E874	E875	E876	E877	E878	E879	E880	E881	E882	E883	E884	E885	E886	E887	E888	E889	E890	E891	E892	E893	E894	E895	E896	E897	E898	E899	E900	E901	E902	E903	E904	E905	E906	E907	E908	E909	E910	E911	E912	E913	E914	E915	E916	E917	E918	E919	E920	E921	E922	E923	E924	E925	E926	E927	E928	E929	E930	E931	E932	E933	E934	E935	E936	E937	E938	E939	E940	E941	E942	E943	E944	E945	E946	E947	E948	E949	E950	E951	E952	E953	E954	E955	E956	E957	E958	E959	E960	E961	E962	E963	E964	E965	E966	E967	E968	E969	E970	E971	E972	E973	E974	E975	E976	E977	E978	E979	E980	E981	E982	E983	E984	E985	E986	E987	E988	E989	E990	E991	E992	E993	E994	E995	E996	E997	E998	E999	E1000	E1001	E1002	E1003	E1004	E1005	E1006	E1007	E1008	E1009	E1010	E1011	E1012	E1013	E1014	E1015	E1016	E1017	E1018	E1019	E1020	E1021	E1022	E1023	E1024	E1025	E1026	E1027	E1028	E1029	E1030	E1031	E1032	E1033	E1034	E1035	E1036	E1037	E1038	E1039	E1040	E1041	E1042	E1043	E1044	E1045	E1046	E1047	E1048	E1049	E1050	E1051	E1052	E1053	E1054	E1055	E1056	E1057	E1058	E1059	E1060	E1061	E1062	E1063	E1064	E1065	E1066	E1067	E1068	E1069	E1070	E1071	E1072	E1073	E1074	E1075	E1076	E1077	E1078	E1079	E1080	E1081	E1082	E1083	E1084	E1085	E1086	E1087	E1088	E1089	E1090	E1091	E1092	E1093	E1094	E1095	E1096	E1097	E1098	E1099	E1100	E1101	E1102	E1103	E1104	E1105	E1106	E1107	E1108	E1109	E1110	E1111	E1112	E1113	E1114	E1115	E1116	E1117	E1118	E1119	E1120	E1121	E1122	E1123	E1124	E1125	E1126	E1127	E1128	E1129	E1130	E1131	E1132	E1133	E1134	E1135	E1136	E1137	E1138	E1139	E1140	E1141	E1142	E1143	E1144	E1145	E1146	E1147	E1148	E1149	E1150	E1151	E1152	E1153	E1154	E1155	E1156	E1157	E1158	E1159	E1160	E1161	E1162	E1163	E1164	E1165	E1166	E1167	E1168	E1169	E1170	E1171	E1172	E1173	E1174	E1175	E1176	E1177	E1178	E1179	E1180	E1181	E1182	E1183	E1184	E1185	E1186	E1187	E1188	E1189	E1190	E1191	E1192	E1193	E1194	E1195	E1196	E1197	E1198	E1199	E1200	E1201	E1202	E1203	E1204	E1205	E1206	E1207	E1208	E1209	E1210	E1211	E1212	E1213	E1214	E1215	E1216	E1217	E1218	E1219	E1220	E1221	E1222	E1223	E1224	E1225	E1226	E1227	E1228	E1229	E1230	E1231	E1232	E1233	E1234	E1235	E1236	E1237	E1238	E1239	E1240	E1241	E1242	E1243	E1244	E1245	E1246	E1247	E1248	E1249	E1250	E1251	E1252	E1253	E1254	E1255	E1256	E1257	E1258	E1259	E1260	E1261	E1262	E1263	E1264	E1265	E1266	E1267	E1268	E1269	E1270	E1271	E1272	E1273	E1274	E1275	E1276	E1277	E1278	E1279	E1280	E1281	E1282	E1283	E1284	E1285	E1286	E1287	E1288	E1289	E1290	E1291	E1292	E1293	E1294	E1295	E1296	E1297	E1298	E1299	E1300	E1301	E1302	E1303	E1304	E1305	E1306	E1307	E1308	E1309	E1310	E1311	E1312	E1313	E1314	E1315	E1316	E1317	E1318	E1319	E1320	E1321	E1322	E1323	E1324	E1325	E1326	E1327	E1328	E1329	E1330	E1331	E1332	E1333	E1334	E1335	E1336	E1337	E1338	E1339	E1340	E1341	E1342	E1343	E1344	E1345	E1346	E1347	E1348	E1349	E1350	E1351	E1352	E1353	E1354	E1355	E1356	E1357	E1358	E1359	E1360	E1361	E1362	E1363	E1364	E1365	E1366	E1367	E1368	E1369	E1370	E1371	E1372	E1373	E1374	E1375	E1376	E1377	E1378	E1379	E1380	E1381	E1382	E1383	E1384	E1385	E1386	E1387	E1388	E1389	E1390	E1391	E1392	E1393	E1394	E1395	E1396	E1397	E1398	E1399	E1400	E1401	E1402	E1403	E1404	E1405	E1406	E1407	E1408	E1409	E1410	E1411	E1412	E1413	E1414	E1415	E1416	E1417	E1418	E1419	E1420	E1421	E1422	E1423	E1424	E1425	E1426	E1427	E1428	E1429	E1430	E1431	E1432	E1433	E1434	E1435	E1436	E1437	E1438	E1439	E1440	E1441	E1442	E1443	E1444	E1445	E1446	E1447	E1448	E1449	E1450	E1451	E1452	E1453	E1454	E1455	E1456	E1457	E1458	E1459	E1460	E1461	E1462	E1463	E1464	E1465	E1466	E1467	E1468	E1469	E1470	E1471	E1472	E1473	E1474	E1475	E1476	E1477	E1478	E1479	E1480	E1481	E1482	E1483

[0022] (J) The amplified target DNA band was detected by the gel electrophoresis after PCR about results and all the **** picornavirus standard stocks for consideration (31 shares). Moreover, as a result of performing plate hybridization, the cross reaction was not accepted between the magnification DNA of each human serum protein type origin as the joint pattern shown in the 1st table. It became clear from

this joint pattern for detection of enterovirus and discernment of each human serum protein type to be possible.

[0023] Example 2 It experimented using the enterovirus separation stock with which it dissociated from the patient of (Discernment A) use microorganism following of detection of an enterovirus separation stock, and a human serum protein type, and the human serum protein type was identified by the protection test using the specific antiserum, and the standard stock of an example 1.

(1) Enterovirus separation stock [Table 3]

株名 (血清型)	分離時期
コクサッキーA群ウイルス4型 (A4)	
1155/72	1972年
1361/82	1982年
0269/84	1984年
0025/86	1986年
0023/87	1987年
0406/89	1989年
0313/91	1991年
エコーウイルス11型 (E11)	
1036/71	1971年
1183/77	1977年
1149/87	1987年
3137/81	1981年
1303/83	1983年
0798/84	1984年
0400/85	1985年
0107/90	1990年
エンテロウイルス71型 (E71)	
ナゴヤ/70	1970年
3059/78	1978年
3359/83	1983年
4132/85	1985年
236a/86	1986年
236c/86	1986年
0253/86	1986年
2587/89	1989年
4094/90	1990年

[0024] (2) Standard stock [Table 4]

コクサッキーA群ウイルス	4型 (A4)
コクサッキーB群ウイルス	2 (B2)
〃	3 (B3)
〃	5 (B5)
エコーウイルス	9 (E9)
〃	11 (E11)
〃	30 (E30)
エンテロウイルス	71 (E71)
ポリオウイルス	3 (PV3)

[0025] (B) the experiment approach and the approach of each virus of the result above to the example 1 given in (B) term -- RNA -- extracting -- an approach given in (** C) term -- every -- cDNA was compounded. Furthermore, as a result of amplifying the gene for solid phase-ized DNA preparation by the approach given in (** E) term, amplifying the gene for DNA probe preparation for human serum protein type discernment by the approach given in (** F) term and performing gel electrophoresis given in (** G) term about these magnification genes DNA, the magnification gene DNA band originating in

all the used stocks has been checked. After refining these magnification gene DNA by the approach given in (** H) term and performing density measurement, plate hybridization was carried out like (** I) term publication, and the binding fraction (%) of each probe was computed. The result is shown in the 2nd table - the 4th table. In addition, the binding fraction of the null column of front Naka is 10% or less of value.

[0026]

[Table 5]

第 2 表

コクサッキーA群ウイルス4型（A4）分離株の型鑑別（結合率：％）

			血清型識別用DNAプローブ							
			1155/72	1361/82	0269/84	0025/86	0023/87	0406/89	0313/91	標準株A4
固 相 化 D N A	分 離 株	1155/72	100							
		1361/82		100						
		0269/84			100	63	50	50	58	
		0025/86			81	100	60	43	50	
		0023/87			50	44	100	36	33	
		0406/89			56	44	36	100	100	
		0313/91			56	44	29	79	100	
	標 準 株	A4								100
		B2								
		B3								
		B5								
		E9								
		E11								
		E30								
		E71								
		PV3								

[0027]

[Table 6]

第 3 表
エコーウイルス 11 型 (E11) 分離株の型鑑別 (結合率 : %)

		血清型識別用 DNA プローブ									
		1036/71	1183/77	1149/78	3137/81	1303/83	0798/84	0400/85	0107/90	標準株 E11	
固 相 化	1036/71	100			37	37		33	23		
	1183/77		100								
	1149/78			100	20	22		20			
	3137/81	43		23	100	111	103	117	92		
	1303/83	33		20	73	100	76	108	81		
	0798/84	20			93	100	100	104	81		
	0400/85	33		20	80	93	76	100	77		
D N A		23			67	78	62	79	100		
		A4									
		B2									
		B3									
		B5									
		E9									
		E11								100	
		E30									
		E71									
		PV3									

[0028]

[Table 7]

第4表
エンテロウイルス71型(E71)分離株の型鑑別(結合率:%)

血清型識別用DNAプローブ		標準株E71									
固相化	E71分離株	71ヤ/70	3059/78	3359/83	4132/85	236a/86	236c/86	0253/86	2587/89	4094/90	標準株E71
		100	110	100	84	98	90	89			
		75	100	64	105	75	62	63			
		82	85	100	100	82	83	85	22		
		79	95	73	100	71	69	63	26	38	
		82	90	91	84	100	97	93			
		89	100	100	58	104	100	100			
		82	90	95	84	104	93	100	100	119	
				32	37				78	100	
					37						
DNA		A4									100
A		B2									
		B3									
		B5									
		E9									
		E11									
		E30									
		E71									
		PV3									

[0029] The cross reaction was not accepted between the same human serum protein types between solid phase-ized DNA of the standard stock origin of all the DNA probes and each human serum protein types of the used enterovirus separation stock a passage clear from the joint pattern shown in the 2nd table - the 4th table. On the other hand, about the separation stock in each human serum protein type, the high cross reaction was accepted on the epidemia viral isolation stock (between the same human serum protein types) separated within about ten years. The gene field where a human serum protein type has a

specific base sequence in said human serum protein type of a known epidemic enterovirus separation stock (stock separated within about ten years) from the above result was amplified, and when performing hybridization using the DNA probe for human serum protein type discernment obtained, it became clear for detection of epidemic enterovirus and discernment of a human serum protein type to be easily possible.

[Translation done.]

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特許法第30条第1項適用申請有り 平成 4 年10月28日～10月30日、日本ウイルス学会主催の「第40回日本ウイルス学会総会」にて文書をもって発表				
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(54)【発明の名称】 エンテロウイルスの検出および識別方法

(57)【要約】

【構成】 (i) エンテロウイルスの5'-非翻訳領域の一部、V p 4 とV p 2 蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、エンテロウイルスの5'-非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つV p 4 およびV p 2 蛋白の一部をコードする遺伝子領域を増幅し、(ii) 該増幅遺伝子DNAを検出することを特徴とするエンテロウイルスの検出法。

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【特許請求の範囲】

【請求項1】 (1) エンテロウイルスの5'-非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、エンテロウイルスの5'-非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域を増幅し、

(ii) 該増幅遺伝子DNAを検出することを特徴とするエンテロウイルスの検出法。

【請求項2】 (1) 血清型が未知のエンテロウイルス分離株の5'-非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、血清型が未知のエンテロウイルス分離株の5'-非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域を増幅し、該増*

CTACTTTGGGTGTCCTGTT (1)

で示される塩基配列を有し、下流の型共通部分に相補性※ ※を有するオリゴヌクレオチドが次の配列

TGGTGGTGGAAAGTTGCCTGA (2)

で示される配列を有するオリゴヌクレオチドであることを特徴とする請求項1又は2のエンテロウイルスの検出または識別方法。

【発明の詳細な説明】

【0001】

【産業上の利用分野】本発明は、エンテロウイルスを高感度に検出し、血清型を識別する方法に関する。

【0002】

【従来の技術】ピコルナウイルス科(Picornaviridae)に属するエンテロウイルス(Enterovirus)はおおよそ70種類の血清型、同じくピコルナウイルス科に属するライノウイルス(Rhinovirus)はおおよそ100種類の血清型に分類されており、多彩な感染症を示し、臨床症状から原因となるウイルスを推定することは困難である。そのため、病原体を確定するにはウイルスの分離同定が必要となる。しかし、現在のエンテロウイルス分離同定法は、培養法を用いてウイルスを分離し、同定のためには更に中和試験が必要になる。そしてこれらウイルスの分

* 増幅遺伝子DNAをマイクロプレートに固

(i) 血清型が既知の流行エンテロウイルス-非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分に相補性を有するオリゴヌクレオチドをプライマーとして用い、血清型が既知の流行エンテロウイルスの5'-非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つVp4蛋白の一部をコードする遺伝子領域を増幅し、血清型識別用DNAプローブとし、

(ii) 該DNAプローブを上記(1)のマイクロプレートに加えて、峻厳条件下でハイブリゼーションさせ、結合プローブの種類を解離とするエンテロウイルスの血清型識別

【請求項3】 (1) エンテロウイルスの5'-非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分に相補性を有するオリゴヌクレオチドが次の配列(1)

既述のようにエンテロウイルスの遺伝子領域内、Vp4とVp2蛋白をコードする塩基配列に相補性を有するプライマーを用いて、エンテロウイルスを検出されている。また、エンテロウイルスの血清型を識別することができ、高い精度でエンテロウイルスを検出し、血清型を識別可能な方法が求められている。

5. J. Clinical microbiology., 28:431-435, 1990; Olive, D., M., 5 J. general Virology, 7(1990)〕。しかしながら、これらの方ではエンテロウイルスの血清型を識別することができ、高い精度でエンテロウイルスを検出し、血清型を識別可能な方法が求められている。

【0003】

【0005】かくして、本発明によれば、

- ＊ マーとして用い、血清型が未知のエンテロ株の5' - 非翻訳領域の一部とエンテロ型に特異的な塩基配列を持つV p 4 およびV p 2の一部をコードする遺伝子領域を増幅し、³²P-DNAをマイクロプレートに固相化し、（i）既知の流行エンテロウイルスの分離株の5' - 非翻訳領域の一部、V p 4 とV p 2 蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型特異的な塩基配列を持つV p 4 およびV p 2 蛋白の一部をコードする遺伝子領域を増幅および標識し、（ii）DNAプローブとし、（iii）DNAプローブ（i）のDNA固相化マイクロプレートに付着した条件下でハイブリダイゼーションさせ、（iv）塩類を解析することとを特徴とするエンテロウイルスの血清型識別方法

- CTACTTTGGGTGTCCGTGTT (1)

で示される塩基配列を有し、下流の型共通部分に相補性※ ※を有するオリゴヌクレオチドが、次の配

TGGTGGTGG AAGTTGCC TGA (2)

セッションで結合させ、結合したブロー
することにより、エンテロウイルスを検
このエンテロウイルスの血清型を識別す
このような方法により、エンテロウイル
検出することができると共に、エンテロ
型を識別することが可能となる。

30 このような方法により、エンテロウイルスを検出することができると共に、エンテロ型を識別することが可能となる。

【0007】エンテロウイルスは、血清型あり、また各血清型間が近縁なため、ハイブリダイゼーション条件では血清型の識別が血清型の識別に際しては、本発明で用いるハイブリダイゼーションを用いるのが好で、厳密条件下でのハイブリダイゼーションアミドの存在下でのハイブリダイゼーションものである。このハイブリダイゼーション

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ルス分離株（すなわち過去10年以内に流行し分離されたエンテロウイルス株）を用いて作成された血清型識別用DNAプローブを用いて、上記峻厳条件下でハイブリダイゼーションを行い、結合パターンを解析することにより、各エンテロウイルスの検出および血清型の識別が可能となる。

【0008】エンテロウイルスの血清型特異的塩基配列を含む遺伝子領域、すなわち「エンテロウイルスの5'-非翻訳領域の一部とエンテロウイルスの血清型に特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域」の増幅は次のとおり行うことができる。まず、診察時に採取した髄液等の臨床検体、臨床検体からの分離培養株、継代培養されている血清型が既知のエンテロウイルス標準株等から常法によりRNAを抽出し、この抽出RNAを逆転写酵素を用いcDNAを作製する。このcDNAに血清型特異的塩基配列を有するオリゴヌクレオチド、すなわち「エンテロウイルス分離株の5'-非翻訳領域の一部、Vp4とVp2蛋白の一部をコードする遺伝子領域の上流の型共通部分および下流の型共通部分に相補性を有するオリゴヌクレオチド」をプライマーとして加えて、エンテロウイルスの5'-非翻訳領域、Vp4とVp2をコードする遺伝子領域を含む長さが約650塩基の遺伝子DNA領域を増幅する。遺伝子の増幅は、通常用いられるPCR法（この*

*PCR法の詳細については、特開昭61号公報、特開昭62-281号公報、Sal 239巻、p487-491参照）により容易に行る。

【0009】エンテロウイルスの血清型を含む遺伝子領域の増幅に際して、ブラ
いることができるオリゴヌクレオチドと
特異的塩基配列を含む遺伝子領域の上流
および下流の型共通部分に相補性を有する
チド、すなわち「エンテロウイルスの5
の一部、Vp4とVp2蛋白の一部をコ
領域の上流の型共通部分および下流の型
性を有するオリゴヌクレオチド」を同時
れば、いかなるオリゴヌクレオチドであ
れらの中で、好ましくは既知の血清型特
ータをもとに、エンテロウイルスに特異
共通性の高い塩基配列を5'-非翻訳領
通部分）とVp2領域（下流の型共通部
その塩基配列に基づいて化学合成したオ
ドをプライマーとして用いるのが適当で、
【0010】化学合成したプライマー、
ロウイルス特異的遺伝子領域の上流の型
性を有するオリゴヌクレオチドとしては、
(1)

CTACTTTGGGTGTCCGTGTT (1)

下流の型共通部分に相補性を有するオリゴヌクレオチド※ ※が下記配列(2)

TGGTGGTGGAAAGTTGCCTGA (2)

で示される塩基配列を有するプライマーを用いるのがより好ましい。上述したプライマーの化学合成は、それ自体既知の通常用いられる核酸合成機、例えばアブライド・バイオシステム社製、モデル381-A DNA合成機等を用いる固相合成法により容易に行うことができる。上記の如くしてPCR法により増幅したエンテロウイルスの血清型特異的塩基配列を含む遺伝子領域DNAは、通常用いられるポリアクリルアミドゲル電気泳動、アガロースゲル電気泳動等により分離し、バンドとして検出することができ、これによりエンテロウイルス由来の遺伝子DNAを確認することができる。なお電気泳動後のDNAバンドの検出は、エチジウム・ブロマ이드で染色し、紫外線照射により容易に行うことができる。

の一部をコードする遺伝子領域」のDN
標識して血清型識別用DNAプローブと
る。この血清型識別用DNAプローブの
ば、DNA増幅反応に用いるdTTPの
dUTPに変更して用いて、DNA増幅
り容易に実施できる。

【0012】かくして得られる各種の血
Aプローブを変性させた後、上記固相化
ルDNA)に加えて、前記峻厳条件下で
ーションさせ、固相化DNAへ結合した
NAプローブの種類および量を、酵素標
用いて検出することにより、固相化DN
NA)の調製に用いたエンテロウイルス

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血清を用いた中和試験で血清型が同定されている標準株である。

【0014】

【表1】

株名（血清型）		略号
コクサッキーA群ウイルス	2型	A2
〃	3〃	A3
〃	4〃	A4
〃	8〃	A8
〃	9〃	A9
コクサッキーB群ウイルス	1型	B1
〃	2〃	B2
〃	3〃	B3
〃	4〃	B4
〃	5〃	B5
〃	6〃	B6
エコーウイルス	3型	E3
〃	4〃	E4
〃	5〃	E5
〃	6〃	E6
〃	9〃	E9
〃	11〃	E11
〃	14〃	E14
〃	16〃	E16
〃	18〃	E18
〃	19〃	E19
〃	24〃	E24
〃	25〃	E25
〃	27〃	E27
〃	30〃	E30
エンテロウイルス	71型	E71
ポリオウイルス	1型	PV1
〃	2〃	PV2
〃	3〃	PV3
ライノウイルス	3型	RH3
〃	7〃	RH7

*【0015】(B) RNAの抽出

上記各ウイルス液を15%シュークロー、
操作により沈殿させた後、その沈殿物を
回収し、フェノール/クロロホルム抽出
ール沈殿を行った。

(C) cDNAの合成

前記(B)項で得た各RNAを鋳型とし、
ンスクリプターゼ(Bethesda Research L.
用いて、各ウイルスに由来するcDNA、

10 【0016】(D) PCR用プライマー

前記(A)項のヒコルナウイルスの遺伝、
できるプライマーペアーを、血清型特異
持つVp4及びVp2蛋白をコードする、
基配列をもとに、5'-非翻訳領域とV
に相補性を有する下記配列(1)および

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*

CTACTTTGGGTGTCGGTGTT (1)

TGGTGGTGGGAAGTTGCCTGA (2)

の塩基配列で示される20塩基のプライマーを、ホスホ
アミダイト(Phosphoramidite)法によりアブライド・バ
イオシステム社製、モデル381-A DNA合成機を
用いて合成し、OPC_{TM}カートリッジを用いて精製し、
PCRのプライマーとして使用した。

【0017】(E) 同相化DNA調製用遺伝子(サンプ

℃30秒、アニーリング工程を45℃1
工程を72℃2分に設定し、アンブリフ
システム(amplification system)シ
て、標的DNAを35サイクル増幅した、
子を同相化用サンプルDNAとして用い、

40 【0018】(F) 血清型識別用DNA

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調製した。1サイクルは、塩基鎖の変性工程を95℃30秒、アニーリング工程を45℃1分、塩基鎖伸長工程を72℃2分に設定し、アンプリフィケーション システム（シータス社）を用いて標的DNAを35サイクル増幅した。このピオチンで標識された遺伝子DNAを血清型識別用DNAプローブとして用いた。

【0019】(G)ゲル電気泳動法による増幅遺伝子DNAの確認

3. 0%のアガロースゲルにエチジウムブロマイドを0.5μg/ml加え、上記(E)および(F)項で増幅したDNAの電気泳動を行った。泳動後254nmの紫外線を照射し、エチジウムブロマイドの発色反応によりDNAバンドを検出し、エンテロウイルスの5'-非翻訳領域の一部と血清型に特異的な塩基配列を持つVp4およびVp2蛋白の一部をコードする遺伝子領域に由来する約650塩基の標的DNAバンドを確認した。

(H)増幅DNAの精製および濃度測定

前記(E)および(F)項で増幅した遺伝子DNAをフェノール/クロロホルムにて抽出後、エタノールを用いて沈殿させ回収し、濃度を260nmの吸光度により算出した。

【0020】(I)プレートハイブリダイゼーション
マイクロプレート固相法(Inouye Hondo, J. Clin. Microbiol. 28:1459, 1990)の変法により行った。上記

(H)項で精製したサンプルDNAを熱変性後、50ng/100μl/wellを、1.5M NaCl、10mMリン酸ナトリウム、10mM EDTA存在下でマイクロプレート(NUNC-IMMUNO PLATE MAXISRP F96)に37℃2時間で固相化した。これをPBS-Tween 20で3回洗浄し、未反応サンプルDNAを除去した。ハイブリダイゼーシ

ョンは前記(H)項で精製した血清型識別用DNAプローブを熱変性後、1.25ng/100μl%ホルムアミド、0.75M NaCl、0.2% Salmon sperm 50μg/mlの存在下でマイクロプレートに50℃8時間行った。ハイブリオン後、マイクロプレートをPBS-Tween 20で3回洗浄し、未反応血清型識別用DNAプローブを除去した。次にペルオキシダーゼ標識ストレプトavidin 1:1, 0.005%洗液(1% BSA, 0.05% X-100, PBS-Tween 20)を滴加し反応させた。再びマイクロプレートをPBS-Tween 20で3回洗浄後、0.012% H₂O₂, 0.05% ルトフェニレンジアミン, 0.05% 過酸化ナトリウム-クエン酸(pH5.0)を添加し、室温で30分、遮光し、4N 硫酸50μl/wellを加え反応によって生じたマイクロプレートの着色を92nmで吸光度(OD)を測定した。各ウェルの吸光度から血清型識別用DNAプローブの結合率(%)を次のとおり求めた。

結合率(%) = (互に異なる血清型ウイルスDNAと識別用DNAプローブとのハイブリダのOD値 ÷ 同一血清型ウイルス由来の識別用DNAプローブとのハイブリダOD値) × 100。

その結果を第1表に示す。なお、第1表いずれも結合10%未満の値である。

【0021】

【表2】

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第1表
標準材の四選別（割合は：％）

		別 用 プ ロ ッ																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
		A1	A2	A3	A4	A5	A6	A7	B1	B2	B3	B4	B5	B6	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12	E13	E14	E15	E16	E17	E18	E19	E20	E21	E22	E23	E24	E25	E26	E27	E28	E29	E30	E31	E32	E33	E34	E35	E36	E37	E38	E39	E40	E41	E42	E43	E44	E45	E46	E47	E48	E49	E50	E51	E52	E53	E54	E55	E56	E57	E58	E59	E60	E61	E62	E63	E64	E65	E66	E67	E68	E69	E70	E71	E72	E73	E74	E75	E76	E77	E78	E79	E80	E81	E82	E83	E84	E85	E86	E87	E88	E89	E90	E91	E92	E93	E94	E95	E96	E97	E98	E99	E100	E101	E102	E103	E104	E105	E106	E107	E108	E109	E110	E111	E112	E113	E114	E115	E116	E117	E118	E119	E120	E121	E122	E123	E124	E125	E126	E127	E128	E129	E130	E131	E132	E133	E134	E135	E136	E137	E138	E139	E140	E141	E142	E143	E144	E145	E146	E147	E148	E149	E150	E151	E152	E153	E154	E155	E156	E157	E158	E159	E160	E161	E162	E163	E164	E165	E166	E167	E168	E169	E170	E171	E172	E173	E174	E175	E176	E177	E178	E179	E180	E181	E182	E183	E184	E185	E186	E187	E188	E189	E190	E191	E192	E193	E194	E195	E196	E197	E198	E199	E200	E201	E202	E203	E204	E205	E206	E207	E208	E209	E210	E211	E212	E213	E214	E215	E216	E217	E218	E219	E220	E221	E222	E223	E224	E225	E226	E227	E228	E229	E230	E231	E232	E233	E234	E235	E236	E237	E238	E239	E240	E241	E242	E243	E244	E245	E246	E247	E248	E249	E250	E251	E252	E253	E254	E255	E256	E257	E258	E259	E260	E261	E262	E263	E264	E265	E266	E267	E268	E269	E270	E271	E272	E273	E274	E275	E276	E277	E278	E279	E280	E281	E282	E283	E284	E285	E286	E287	E288	E289	E290	E291	E292	E293	E294	E295	E296	E297	E298	E299	E300	E301	E302	E303	E304	E305	E306	E307	E308	E309	E310	E311	E312	E313	E314	E315	E316	E317	E318	E319	E320	E321	E322	E323	E324	E325	E326	E327	E328	E329	E330	E331	E332	E333	E334	E335	E336	E337	E338	E339	E340	E341	E342	E343	E344	E345	E346	E347	E348	E349	E350	E351	E352	E353	E354	E355	E356	E357	E358	E359	E360	E361	E362	E363	E364	E365	E366	E367	E368	E369	E370	E371	E372	E373	E374	E375	E376	E377	E378	E379	E380	E381	E382	E383	E384	E385	E386	E387	E388	E389	E390	E391	E392	E393	E394	E395	E396	E397	E398	E399	E400	E401	E402	E403	E404	E405	E406	E407	E408	E409	E410	E411	E412	E413	E414	E415	E416	E417	E418	E419	E420	E421	E422	E423	E424	E425	E426	E427	E428	E429	E430	E431	E432	E433	E434	E435	E436	E437	E438	E439	E440	E441	E442	E443	E444	E445	E446	E447	E448	E449	E450	E451	E452	E453	E454	E455	E456	E457	E458	E459	E460	E461	E462	E463	E464	E465	E466	E467	E468	E469	E470	E471	E472	E473	E474	E475	E476	E477	E478	E479	E480	E481	E482	E483	E484	E485	E486	E487	E488	E489	E490	E491	E492	E493	E494	E495	E496	E497	E498	E499	E500	E501	E502	E503	E504	E505	E506	E507	E508	E509	E510	E511	E512	E513	E514	E515	E516	E517	E518	E519	E520	E521	E522	E523	E524	E525	E526	E527	E528	E529	E530	E531	E532	E533	E534	E535	E536	E537	E538	E539	E540	E541	E542	E543	E544	E545	E546	E547	E548	E549	E550	E551	E552	E553	E554	E555	E556	E557	E558	E559	E560	E561	E562	E563	E564	E565	E566	E567	E568	E569	E570	E571	E572	E573	E574	E575	E576	E577	E578	E579	E580	E581	E582	E583	E584	E585	E586	E587	E588	E589	E590	E591	E592	E593	E594	E595	E596	E597	E598	E599	E600	E601	E602	E603	E604	E605	E606	E607	E608	E609	E610	E611	E612	E613	E614	E615	E616	E617	E618	E619	E620	E621	E622	E623	E624	E625	E626	E627	E628	E629	E630	E631	E632	E633	E634	E635	E636	E637	E638	E639	E640	E641	E642	E643	E644	E645	E646	E647	E648	E649	E650	E651	E652	E653	E654	E655	E656	E657	E658	E659	E660	E661	E662	E663	E664	E665	E666	E667	E668	E669	E670	E671	E672	E673	E674	E675	E676	E677	E678	E679	E680	E681	E682	E683	E684	E685	E686	E687	E688	E689	E690	E691	E692	E693	E694	E695	E696	E697	E698	E699	E700	E701	E702	E703	E704	E705	E706	E707	E708	E709	E710	E711	E712	E713	E714	E715	E716	E717	E718	E719	E720	E721	E722	E723	E724	E725	E726	E727	E728	E729	E730	E731	E732	E733	E734	E735	E736	E737	E738	E739	E740	E741	E742	E743	E744	E745	E746	E747	E748	E749	E750	E751	E752	E753	E754	E755	E756	E757	E758	E759	E760	E761	E762	E763	E764	E765	E766	E767	E768	E769	E770	E771	E772	E773	E774	E775	E776	E777	E778	E779	E780	E781	E782	E783	E784	E785	E786	E787	E788	E789	E790	E791	E792	E793	E794	E795	E796	E797	E798	E799	E800	E801	E802	E803	E804	E805	E806	E807	E808	E809	E810	E811	E812	E813	E814	E815	E816	E817	E818	E819	E820	E821	E822	E823	E824	E825	E826	E827	E828	E829	E830	E831	E832	E833	E834	E835	E836	E837	E838	E839	E840	E841	E842	E843	E844	E845	E846	E847	E848	E849	E850	E851	E852	E853	E854	E855	E856	E857	E858	E859	E860	E861	E862	E863	E864	E865	E866	E867	E868	E869	E870	E871	E872	E873	E874	E875	E876	E877	E878	E879	E880	E881	E882	E883	E884	E885	E886	E887	E888	E889	E890	E891	E892	E893	E894	E895	E896	E897	E898	E899	E900	E901	E902	E903	E904	E905	E906	E907	E908	E909	E910	E911	E912	E913	E914	E915	E916	E917	E918	E919	E920	E921	E922	E923	E924	E925	E926	E927	E928	E929	E930	E931	E932	E933	E934	E935	E936	E937	E938	E939	E940	E941	E942	E943	E944	E945	E946	E947	E948	E949	E950	E951	E952	E953	E954	E955	E956	E957	E958	E959	E960	E961	E962	E963	E964	E965	E966	E967	E968	E969	E970	E971	E972	E973	E974	E975	E976	E977	E978	E979	E980	E981	E982	E983	E984	E985	E986	E987	E988	E989	E990	E991	E992	E993	E994	E995	E996	E997	E998	E999	E1000	E1001	E1002	E1003	E1004	E1005	E1006	E1007	E1008	E1009	E1010	E1011	E1012	E1013	E1014	E1015	E1016	E1017	E1018	E1019	E1020	E1021	E1022	E1023	E1024	E1025	E1026	E1027	E1028	E1029	E1030	E1031	E1032	E1033	E1034	E1035	E1036	E1037	E1038	E1039	E1040	E1041	E1042	E1043	E1044	E1045	E1046	E1047	E1048	E1049	E1050	E1051	E1052	E1053	E1054	E1055	E1056	E1057	E1058	E1059	E1060	E1061	E1062	E1063	E1064	E1065	E1066	E1067	E1068	E1069	E1070	E1071	E1072	E1073	E1074	E1075	E1076	E1077	E1078	E1079	E1080	E1081	E1082	E1083	E1084	E1085	E1086	E1087	E1088	E1089	E1090	E1091	E1092	E1093	E1094	E1095	E1096	E1097	E1098	E1099	E1100	E1101	E1102	E1103	E1104	E1105	E1106	E1107	E1108	E1109	E1110	E1111	E1112	E1113	E1114	E1115	E1116	E1117	E1118	E1119	E1120	E1121	E1122	E1123	E1124	E1125	E1126	E1127	E1128	E1129	E1130	E1131	E1132	E1133	E1134	E1135	E1136	E1137	E1138	E1139	E1140	E1141	E1142	E1143	E1144	E1145	E1146	E1147	E1148	E1149	E1150	E1151	E1152	E1153	E1154	E1155	E1156	E1157	E1158	E1159	E1160	E1161	E1162	E1163	E1164	E1165	E1166	E1167	E1168	E1169	E1170	E1171	E1172	E1173	E1174	E1175	E1176	E1177	E1178	E1179	E1180	E1181	E1182	E1183	E1184	E1185	E1186	E1187	E1188	E1189	E1190	E1191	E1192	E1193	E1194	E1195	E1196	E1197	E1198	E1199	E1200	E1201	E1202	E1203	E1204	E1205	E1206	E1207	E1208	E1209	E1210	E1211	E1212	E1213	E1214	E1215	E1216	E1217	E1218	E1219	E1220	E1221	E1222	E1223	E1224	E1225	E1226	E1227	E1228	E1229	E1230	E1231	E1232	E1233	E1234	E1235	E1236	E1237	E1238	E1239	E1240	E1241	E1242	E1243	E1244	E1245	E1246	E1247	E1248	E1249	E1250	E1251	E1252	E1253	E1254	E1255	E1256	E1257	E1258	E1259	E1260	E1261	E1262	E1263	E1264	E1265	E1266	E1267	E1268	E1269	E1270	E1271	E1272	E1273	E1274	E1275	E1276	E1277	E1278	E1279	E1280	E1281	E1282	E1283	E1284	E1285	E1286	E1287	E1288	E1289	E1290	E1291	E1292	E1293	E1294	E1295	E1296	E1297	E1298	E1299	E1300	E1301	E1302	E1303	E1304	E1305	E1306	E1307	E1308	E1309	E1310	E1311	E1312	E1313	E1314	E1315	E1316	E1317	E1318	E1319	E1320	E1321	E1322	E1323	E1324	E1325	E1326	E1327	E1328	E1329	E1330	E1331	E1332	E1333	E1334	E1335	E1336	E1337	E1338	E1339	E1340	E1341	E1342	E1343	E1344	E1345	E1346	E1347	E1348	E1349	E1350	E1351	E1352	E1353	E1354	E1355	E1356	E1357	E1358	E1359	E1360	E1361	E1362	E1363	E1364	E1365	E1366	E1367	E1368	E1369	E1370	E1371	E1372	E1373	E1374	E1375	E1376	E1377	E1378	E1379	E1380	E1381	E1382	E1383	E1384	E1385	E1386	E1387	E1388	E1389	E1390	E1391	E1392	E1393	E1394	E1395	E1396	E1397	E1398	E1399	E1400	E1401	E1402	E1403	E1404	E1405	E1406	E1407	E1408	E1409	E1410	E1411	E1412	E1413	E1414	E1415	E1416	E1417	E1418	E1419	E1420	E1421	E1422	E1423	E1424	E1425	E1426	E1427	E1428	E1429	E1430	E1431	E1432	E1433	E1434	E1435	E1436	E1437	E1438	E1439	E1440	E1441	E1442	E1443	E1444	E1445	E1446	E1447	E1448	E1449	E1450	E1451	E1452	E1453	E1454	E1455	E1456	E1457	E1458	E1459	E1460	E1461	E1462	E1463	E1464	E1465	E1466	E1467	E1468	E1469	E1470	E1471	E1472	E1473	E1474	E1475	E1476	E1477	E1478	E1479	E1480	E1481	E1482	E1483	E1484	E1485	E1486	E1487	E1

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株名 (血清型)	分離時期
コクサッキーA群ウイルス4型 (A4)	
1155/72	1972年
1381/82	1982年
0269/84	1984年
0025/86	1986年
0023/87	1987年
0406/89	1989年
0313/91	1991年
エコーウイルス11型 (E11)	
1036/71	1971年
1183/77	1977年
1148/87	1987年
3137/81	1981年
1303/83	1983年
0798/84	1984年
0400/85	1985年
0107/90	1990年
エンテロウイルス71型 (E71)	
ナグヤ/70	1970年
3059/78	1978年
3359/83	1983年
4132/85	1985年
236a/86	1986年
236c/86	1986年
0253/86	1986年
2587/89	1989年
4094/90	1990年

【0024】(2) 標準株
【表4】

コクサッキーA群ウイルス	4型 (A4)
コクサッキーB群ウイルス	2 (B2)
〃	3 (B3)
〃	5 (B5)
エコーウイルス	9 (E9)
〃	11 (E11)
〃	30 (E30)
エンテロウイルス	71 (E71)
ポリオウイルス	3 (PV3)

【0025】(B) 実験方法および結果
上記の各ウイルスから実施例1の(B)項記載の方法に

よりRNAを抽出し、同(C)項記載のAを合成した。更に同(E)項記載の方
A調製用遺伝子を増幅し、同(F)項記
型識別用DNAプローブ調製用遺伝子を
の増幅遺伝子DNAについて同(G)項
泳動を行った結果、用いた全ての株に由
子DNAバンドが確認できた。これら増
を同(H)項記載の方法で精製し、濃度
に、同(I)項記載と同様にプレートハ
クションさせ、各プローブの結合率(%)
の結果を第2表～第4表に示す。なお、
結合率が10%以下の値である。

【0026】
【表5】

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第2表

コクサッキーA群ウイルス4型(A4)分離株の型鑑別(結合率:%)

			血清型識別用DNAプローブ						
			1155/72	1361/82	0269/84	0025/86	0023/87	0406/89	0313/91
固 相 化 D N A	A 4 分 離 株	1155/72	100						
		1361/82		100					
		0269/84			100	53	50	50	58
		0025/86			81	100	60	43	50
		0023/87			50	44	100	36	33
		0406/89			56	44	36	100	100
		0313/91			56	44	29	79	100
	標 準 株	A4							
		B2							
		B3							
		B5							
		E9							
		E11							
		E30							
		E71							
		PV3							

【0027】

【表6】

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第3表
エコーウイルスII型(E11)分離株の型鑑別(結合率:%)

血清型鑑別用DNAプローブ			1036/71	1183/77	1149/78	3137/81	1303/83	0798/84	0400/85	0107/90	標準株E11
固相化DNA	E11分離株	1036/71	100			37	37		33	23	
		1183/77		100							
		1149/78			100	20	22		20		
		3137/81	43		23	100	111	103	117	92	
		1303/83	38		20	73	100	75	108	81	
		0798/84	20			93	100	100	104	81	
		0400/85	33		20	80	93	76	100	77	
標準株	A4 B2 B3 B5 E9 E11 E30 E71 PV3	0107/90	23			67	78	62	79	100	
											100

【0028】

【表7】

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[illegible]

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